Biochimica et Biophysica Acta 818 (1985) 310–315
Elsevier

BBA 72721

Aged human erythrocytes exhibit increased anion exchange

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(Received April 11th, 1985)

Key words: Anion transport; Aging; Erythrocyte membrane; Membrane permeability

Young and old erythrocytes show different rate constants of anion exchange as measured by $^{35}SO_4^{2-}$ efflux at 37°C. Results indicate that the rate constant for $^{35}SO_4^{2-}$ efflux (SO_4^{2-}-Cl^- exchange) from old cells is approximately 20% greater than from young less dense cells. The cell water volume of older cells is also decreased. Based on these results and previously reported decreases of cell membrane area in aged cells we conclude that anion exchange ($^{35}SO_4^{2-}$) is increased in older, more dense human erythrocytes.

Introduction

The mammalian erythrocyte possesses a finite lifespan. As a consequence of aging in vivo or in vitro, biochemical, functional, and structural properties of the erythrocyte membrane are altered. For example, membrane enzymes such as acetylcholinesterase [1], adenylate cyclase and protein kinase [2] have been reported to show an age-related decrease in activity. Other studies have shown decreases in membrane phospholipids and cholesterol [1,3–5], crosslinking of membrane proteins [6,7], vesiculation of the plasma membrane [5,8] and exposure of an age-related antigen which appears to aid in the removal of senescent cells from the circulation [9,10]. Furthermore, this 'senescent cell antigen' appears to crossreact with band 3 [11], the protein responsible for anion exchange across the erythrocyte membrane [12,13]. An interest in understanding the process of erythrocyte aging and the evidence that band 3 may be the progenitor of the 'senescent cell antigen' have prompted us to investigate whether there is a difference in anion transport in old and young human erythrocytes separated by density centrifugation.

Materials and Methods

Preparation of erythrocytes. Blood was drawn from healthy adult donors, heparinized, centrifuged and the plasma and buffy coat removed. The cells were washed twice with isotonic (300 mosM) buffer (80 mequiv./l NaCl, 5 mequiv./l KCl, 10 mequiv./l Na_2SO_4, 5 mequiv./l Na-Hepes, 5 mM glucose and 100 mM sucrose, adjusted to pH 7.4) [14]. These packed cells were mixed and a sample removed before density separation to be used for control studies.

Age separation of erythrocytes. Cells were separated by age according to the method of Murphy [15]. Briefly, 10 ml aliquots of packed cells adjusted to a hematocrit of approx. 80% were transferred to 16 × 100 mm polycarbonate centrifuge tubes and centrifuged for 60 min at 27000 × *g* in a 34° fixed-angle rotor at 30°C. The centrifugate was then discarded and the top and bottom 10% of the cells were aspirated from the tubes and

Abbreviations: DIDS, 4,4'-diisothiocyanostilbene-2,2'-disulfonate; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

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resuspended in buffer. Subsequent analyses were done on these two fractions and the unseparated sample taken previously. This technique for separating cells has been shown to isolate the oldest cells in the most dense fraction as evidenced by previously established age-related cell characteristics [1,6,16] and ^{59}Fe-labeling studies [12,17–19].

Determination of anion efflux rate constants and permeability coefficients. The anion exchange of density separated and unseparated erythrocytes was studied using methods similar to those of Castranova et al. [14] at pH 7.4 and 37°C. The rate of $^{35}SO_4^{2-}$ efflux (SO_4^{2-}-Cl^- exchange) was carried out in cell suspensions of 6% or less. The rate coefficient, k, for this process was calculated by linear regression analysis of the plot of $\ln(1 - a_t/a_\infty)$ vs. time where a_t and a_∞ indicate the extracellular radioactivity at sampling time t, and at isotopic equilibrium, respectively. The negative of the slope of this plot is equal to the rate coefficient of the $^{35}SO_4^{2-}$ efflux.

The permeability coefficient P is determined from the relationship:

$$P = kVA^{-1} \quad (1)$$

where V is the cell water volume and A is cell membrane area.

DIDS inhibition of anion exchange. Sulfate efflux was also studied under conditions where anion exchange was inhibited with DIDS. Cells were treated with 5 μM DIDS using a procedure following that of Funder et al. [20]. Suspensions were treated with the agent for the last 30 min of the 2.5 h $^{35}SO_4^{2-}$-labeling incubation period and the same concentration of the inhibitor was present in the medium during efflux experiments.

Determination of cell water content. Determinations of cell water content of old (most dense), young and unseparated cells were made by measuring weights of the packed cell samples before and after drying. A correction for trapped extracellular water was made by measuring the extracellular [^{3}H]inulin space in a manner similar to that used by Brahm [21] and Funder and Wieth [22]. The extracellular volume of unseparated packed cells was found to be 2.07% which is in excellent agreement with the 2% value reported by Brahm [21].

Results

Table I presents the efflux rate constants of unseparated and density separated erythrocytes from eight donors of different ages and of both sexes. As can be seen, there is a difference between

TABLE I

SULFATE EFFLUX RATE CONSTANTS OF LEAST DENSE AND MOST DENSE ERYTHROCYTES

Washed cells were separated by density centrifugation. The rate of sulfate efflux was determined from cell suspensions of the top 10% and bottom 10% of the cells (youngest and oldest cells, respectively) as described in the text.

Donor	Age	Sex	$^{35}SO_4^{2-}$ efflux rate constant (k, 10^{-4} s^{-1})			% Difference
			unseparated	least dense (youngest)	most dense (oldest)	
B.F.	26	M	6.37	5.79	6.81	18
K.P.	21	F	5.21	5.16	5.93	15
D.K.	34	M	5.67	5.37	6.42	20
R.T.	23	M	5.47	5.09	5.96	17
D.B.	25	M	5.69	5.37	6.50	21
M.L.	23	F	5.65	5.44	6.20	14
B.G.	42	M	5.77	5.23	6.75	29
T.S.	19	M	4.52	4.37	5.42	24
Mean ± S.E. (n = 8)			5.54 ± 1.86	5.23 ± 1.43 *	6.18 ± 1.95 *	20

* Most-dense and least-dense cells significantly different, $p < 0.001$ (2-sample 2-tailed t-test).

the efflux rate constants of the age separated cells. The older more dense cells exhibit a sulfate efflux rate approx. 20% greater than the least dense fraction. Furthermore, the rate constants of both fractions also differ significantly from the unseparated cells. The least-dense fraction has a mean rate constant less than the unseparated sample while the older cell fraction has a rate constant greater than the unseparated cells.

As can also be seen from Table I, not only are the rate constants for the more-dense older cells greater than that for the younger cells, this difference is seen in the cells of every individual studied.

Furthermore, there appears to be no direct relationship between the difference in rate constants and the age of the individual donors ($r = 0.64$).

The efflux rate constants of density separated cell fractions from two males differing in age by 22 years were compared. The rate constants for old (more-dense) cells from two donors were $(6.26 \pm 0.34) \cdot 10^{-4}\ s^{-1}$ and $(5.75 \pm 0.24) \cdot 10^{-4}\ s^{-1}$ for the older and younger donors, respectively, while the younger cells of the donors gave rate constants of $(4.92 \pm 0.19) \cdot 10^{-4}\ s^{-1}$ and $(4.87 \pm 0.27) \cdot 10^{-4}\ s^{-1}$. There is neither a significant difference in the efflux rate constants between the most dense cells of the two donors, nor is there any difference between the least dense cells of the two individuals. However, the data do show a significant difference in the rate constants between the age separated fractions of each individual.

The efflux rate constants of density separated cells in the presence and absence of 5 μM DIDS were compared. The erythrocytes from two donors were separated by density and efflux measurements conducted as described above. These studies were done to determine whether the difference between the rate constants of old and young cells is a result of a greater non-carrier-mediated efflux (leak) of sulfate in the older cells. There is a 98% inhibition of the rate of $^{35}SO_4^{2-}$ efflux in both the least-dense younger cells and the older fraction of cells to a rate of about $0.1 \cdot 10^{-4}\ s^{-1}$.

Non-inhibited old and young cells show an approximately 20% difference in rate constants, as is seen in Table I.

In light of the fact that the membrane permeability coefficient, P, of erythrocytes is described by Eqn. 1 and thus is proportional to cell volume, it is necessary to determine whether there are differences in the cell water volumes between old and young cells. In order to ascertain whether there are real differences in the anion permeability we have measured intracellular water content of unseparated, old and young human erythrocytes. As is seen in Table II, the oldest cells contain 21% less intracellular water than the younger cells.

TABLE II

WATER CONTENT OF AGE-SEPARATED ERYTHROCYTES

Determinations of cell water content were made by measuring weights of packed cell samples before and after drying. A correction for trapped extracellular water was made by measuring the [^{3}H]inulin space.

Erythrocytes	Cell water (l/kg solids) ± S.E.	Number of experiments
Unseparated	1.73 ± 0.07	11
Youngest	1.81 ± 0.05 *	9
Oldest	1.48 ± 0.04 *	11

* Oldest and youngest cells significantly different, $p < 0.001$ (2-sample 2-tailed t-test).

Discussion

During erythrocyte senescence, membrane properties are altered which include changes in transport associated functions. For example Luthra and Kim [23] observed a decrease in $(Ca^{2+} + Mg^{2+})$-ATPase activity in older cells, which may be due to a decrease in calmodulin induced activity of $(Ca^{2+} + Mg^{2+})$-ATPase, as reported by Ekholm et al. [24]. In addition, decreased ATP levels have been observed in older erythrocytes by numerous investigators [1,3,25–28]. However, the significance of decreased ATP content is not clear in light of the decrease of cell volume resulting in no net change of ATP concentration in old cells as reported by Kirkpatrick et al. [29]. Intracellular ATP, calcium and magnesium appear to be major determinants of cell deformability as shown by Weed et al. [25] who observed a decrease in deformability along with increased intracellular calcium in ATP-depleted cells. La Celle et al. [30] found older cells contain lower ATP levels, more Ca^{2+} and less Mg^{2+} than normal control cells.

The importance of Ca^{2+} in membrane structure and function is well documented and has been shown to affect active [31,32] and passive [33] transport of cations, as well as the anion exchange transport system [34] in unseparated erythrocytes.

The anion exchange mechanism is important in the transport of CO_2 from body tissues and it has been calculated by Wieth [35] to be only marginally sufficient to satisfy the transport needs of a normal individual. Studies by Kee, Jennings and Passow [36] have shown sulfate exchange to parallel chloride and bicarbonate exchange. In addition, both the anion exchange process and the 'senescent cell antigen' have been shown to be associated with the band 3 protein of the red cell membrane in cells aged in vivo and in vitro [12,13].

The older fraction produced by our separation technique shows an increased $^{35}SO_4^{2-}$ efflux rate constant as compared to the younger fraction. This result is a generalized effect in all eight individuals as shown in Table I. The lack of correlation between age and the difference in rate constants suggests that this result is not an acquired phenomena due to stem-cell aging but appears to be related to the circulation age of the cell.

It is interesting to note that Low [37] has shown that incubation of erythrocytes with Ca^{2+} and the ionophore A23187 results in decreased $^{35}SO_4^{2-}$ anion exchange. These results were verified in previous work in our laboratory [38]. Since older cells appear to contain more calcium, one would expect, if anything, a slight inhibition of sulfate exchange. However, our results show this not to be true as the older cells, in fact, show an increased exchange rate. This could be due to a compensatory mechanism of the aged cells resulting from an increased level of intracellular calcium.

Since the efflux of $^{35}SO_4^{2-}$ is the sum of exchange and leak fluxes, a possible explanation for the increased rate of $^{35}SO_4^{2-}$ efflux in the older cells could be an increased leak. However, the possibility of a non-carrier-mediated leak increase is not supported by the experiments which show DIDS inhibition of both young and old red cell anion exchange by 98% to a value of about $0.1 \cdot 10^{-4}$ s^{-1}. If passive leak permeability were increased in older cells, the passive non-inhibitable portion would have to be equal to the 20% difference observed between the two cell fractions which would be $0.91 \cdot 10^{-4}$ S^{-1}.

Another possible explanation for our results resides in the fact that what has been measured in these studies is the rate constant (k) rather than the permeability coefficient (P) for the efflux of the anion. The permeability coefficient of erythrocytes is described by Eqn. 1 ($P = kVA^{-1}$) and the data in Table II show the older cells have an intracellular water volume about 20% less than the younger cells. A decrease in water content of older, more-dense red cell fractions has also been reported by other investigators [1,6,27,39,40]. Consequently, if one assumes that no changes in membrane area have taken place in older cells, the permeability coefficient, a more appropriate measure of the $^{35}SO_4^{2-}$ membrane permeability, would not be different. This results because the 20% decrease in volume would offset the 20% increase in efflux.

However, it is well established that during the aging of erythrocytes, a significant loss of membrane area does take place [1,5,8,16,26,41–46]. This loss appears to be indicative of the process of vesiculation which is seen in erythrocytes aged in vitro [5,8,26,47–50], and in cells treated with Ca^{2+} and ionophore A23187 [50–52].

Cohen et al. [1] have indicated an approximate 17.4% decrease in membrane protein and a 26% loss of phospholipid in human erythrocytes aged in vivo. Furthermore, Halbhuber et al. [5] have shown a lipid loss in stored erythrocytes of 5 to 20%, and Haradin et al. [3] have also shown a 20% loss of lipoid material from stored cells. Thus, the loss of membrane material observed in older erythrocytes could clearly reduce the available membrane surface area in these cells. If we assume that the surface area of aged erythrocytes is decreased by the approximately 20% suggested by the studies mentioned above, then the permeability coefficient in Eqn. 1 would result in a 20% increase in permeability of $^{35}SO_4^{2-}$ in aged cells.

If anion carrier molecules were left in the older erythrocyte membrane when vesiculation (membrane loss) occurred, and these carriers remained functional, it would be possible for the older cells to have an increased rate constant. This possibility is supported by the work of Halbhuber et al. [49] which implies an increase in band 3 in older cells. On the other hand, Fishbeck et al. [46] report that

intramembranous particle density remains constant in aging erythrocytes. In addition, Lutz et al. [47] show that vessicles released from erythrocytes aged in vitro by ATP depletion contain integral protein content similar to intact membrane. In light of these studies, it does not seem likely that the older cells contain more carriers per unit area than young cells.

Another consideration is that there is an increase in crosslinking between proteins in erythrocyte membranes aged in vivo or in vitro [6,7]. While the exact nature of the crosslinked proteins is not known in old red cells, Lorand et al. [53] have shown that erythrocytes exposed to increased intracellular Ca^{2+} form crosslinked membrane protein clusters which involve spectrin, bands 3, 2.1, and 4.1. Low et al. [54] have recently reported that denaturation of hemoglobin in aged cells forms hemichromes which aggregate band 3 into clusters, and that these clusters lead to the exposure of the senescent cell antigen. Thus, it may be that band 3-mediated anion exchange is increased by a crosslinking process. Crosslinkages between membrane proteins could possibly result in an increased number of functional sites or in some way make the environment more functionally favorable for anion exchange in aged erythrocytes.

Increased microviscosity has been observed by spin-label studies in human erythrocytes aged in vivo [27] and also in bovine erythrocytes [4] and it may be that decreased lipid fluidity alters lipid-protein interactions which could increase the anion exchange mechanism. Work by Grunze et al. [55] has shown that decreased membrane cholesterol increases anion exchange in unseparated erythrocytes.

In view of these findings, a decreased level of cholesterol in aged erythrocytes could explain our observation that $^{35}SO_4^{2-}$ efflux is increased in older red blood cells.

Acknowledgement

The secretarial help of Mrs. Arlene Martinez in preparing the manuscript is greatly appreciated.

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